Bovine Herpesvirus 4 Induces Apoptosis of Human Carcinoma Cell Lines \textit{In vitro} and \textit{In vivo}

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\textbf{Abstract}

The idea of using oncolytic viruses for the treatment of cancers was proposed a century ago. During the last two decades, viruses able to replicate specifically in cancer cells and to induce their lysis were identified and were genetically modified to improve their viro-oncolytic properties. More recently, a new approach consisting of inducing selective apoptosis in cancer cells through viral infection has been proposed; this approach has been called viro-oncoapoptosis. In the present study, we report the property of bovine herpesvirus-4 (BoHV-4) to induce, \textit{in vitro} and \textit{in vivo}, apoptosis of some human carcinomas. This conclusion relies on the following observations: (a) \textit{In vitro}, BoHV-4 infection induced apoptosis of A549 and OVCAR carcinoma cell lines in a time- and dose-dependent manner. (b) Apoptosis was induced by the expression of an immediate-early or an early BoHV-4 gene, but did not require viral replication. (c) Cell treatment with caspase inhibitors showed that apoptosis induced by BoHV-4 relied mainly on caspase-10 activation. (d) Infection of cocultures of A549 or OVCAR cells mixed with human 293 cells (in which BoHV-4 does not induce apoptosis) showed that BoHV-4 specifically eradicated A549 or OVCAR cancer cells from the cocultures. (e) Finally, \textit{in vivo} experiments done with nude mice showed that BoHV-4 intratumoral injections reduced drastically the growth of preestablished A549 xenografts. Taken together, these results suggest that BoHV-4 may have potential as a viro-oncoapoptotic agent for the treatment of some human carcinomas. Moreover, further identification of BoHV-4 proapoptotic gene(s) and the cellular pathways targeted by this or these gene(s) could lead to the design of new cancer therapeutic strategies. \cite{Gillet2005}

\textbf{Introduction}

Cancer treatments with novel mechanisms of action without cross-resistance to currently available treatments are needed. The idea of using oncolytic viruses for the treatment of cancers was proposed as early as 1904 based on the observation that tumor regression occurred in some patients after rabies vaccination or viral illness \cite{Gillet2005, Vanderplasschen2005}. During the last two decades, advances in tumor biology, virology, and molecular biology have provided the tools required to identify oncolytic virus candidates, among which some have been genetically modified to improve their oncolytic, immunogenic, and safety properties. Some genetically modified oncolytic viruses are now used as a plausible alternative treatment for some tumors \cite{Vanderplasschen2005}.

More recently, a new approach consisting of inducing selective apoptosis in cancer cells through viral infection by replicative or even defective viruses has been proposed; this approach has been called viro-oncoapoptosis \cite{Gillet2005}. Viro-oncoapoptosis, like other cancer therapeutic approaches, relies on expression of specific cellular genes and, thus, it is impeded by the extreme variety of tumors. Indeed, because different tumor types exhibit very different patterns of gene expression, it is unlikely that a single oncolytic virus strain could be used for efficient treatment of all cancers. In this context, the finding and designing of new oncolytic viruses is needed for the future development of viro-oncoapoptotopic treatment of cancers.

Bovine herpesvirus 4 (BoHV-4) belongs to the \textit{Herpesviridae} family, \textit{\gamma-herpesviridae} subfamily, \textit{rhadinovirus} genus \cite{Gillet2005}. BoHV-4 has been isolated throughout the world from healthy cattle, as well as from those exhibiting a variety of diseases. During lytic infection, BoHV-4 protein expression is temporally regulated. The proteins are classified into three kinetic classes depending on the order of their synthesis during the infection \cite{Gillet2005}. They are expressed chronologically as immediate-early, early, and late proteins. Immediate-early proteins are expressed directly after release of the viral genome from the capsid into the nucleus. Whereas early protein expression occurs after synthesis of immediate-early proteins, late protein expression depends on the expression of both immediate-early and early proteins and on viral DNA replication.

The replication of most \textit{\gamma-herpesviruses} is restricted to their natural host species. BoHV-4 is one of the few exceptions to this rule. Indeed, it has been shown that BoHV-4 is able to replicate in a broad range of host species both \textit{in vivo} and \textit{in vitro}. In addition to cattle, isolates of BoHV-4 have been recovered from other ruminant species \cite{Gillet2005}. Sporadic isolations were also reported in lions, cats, and owl monkeys \textit{(Aotus trivirgatus}; refs. 11, 12). Experimentally, BoHV-4 was also shown to infect goats \cite{Gillet2005}, guinea pigs, and rabbits \cite{Gillet2005}. \textit{In vitro}, BoHV-4 is able to replicate in primary cell cultures or cell lines from a broad spectrum of host species, such as sheep, goats, swine, cats, dogs, rabbits, mink, horses, turkeys, ferrets, chickens, hamsters, rats, mice, and monkeys \cite{Gillet2005, Vanderplasschen2005}. Recently, several studies have shown that some human cell lines support BoHV-4 infection \cite{Gillet2005, Vanderplasschen2005}.

In comparison to other \textit{\gamma-herpesviruses}, BoHV-4 encodes a relatively small number of genes susceptible to affect the biology of the infected cells \cite{Gillet2005}. However, BoHV-4 could interfere with the
cell apoptotic machinery through at least two different mechanisms. Indeed, BoHV-4 possesses two genes that could protect the infected cells from apoptosis: ORF16 and ORF71 encoding a V-FLIP and a v-FLIP, respectively. Products of these two genes have been previously shown to inhibit apoptosis when overexpressed transiently (24, 25). Our recent study showed that nonreplicative infection of human HeLa cells by BoHV-4 protects the infected cells from apoptosis induced by cycloheximide and tumor necrosis factor (TNF)-α treatment. Our recent unpublished data showed that this observed effect was due to BoHV-4-encoded v-FLIP.4 However, despite the presence of these antiapoptotic genes, several studies have shown that the expression of BoHV-4 replication cycle in bovine permissive cells leads to apoptosis of infected cell (26, 27).

In this study, we pursue our investigation on the interactions between BoHV-4 and human cells. We report the property of BoHV-4 to induce in vitro and in vivo apoptosis of some human carcinoma cells. The data presented in this study suggest that BoHV-4 could have potential as a vaso-oncopoapoptotic agent for the treatment of some human carcinomas and that further identification of BoHV-4 proapoptotic gene(s) and the cellular pathways targeted by this or these gene(s) could lead to the design of new cancer therapeutic strategies.

Materials and Methods

Cell lines and virus strains. Human 293T kidney cells [American Type Culture Collection (ATCC), Manassas, VA], A549 lung carcinoma cells (ATCC), and Madin-Darby bovine kidney cells (ATCC) were cultured in MEM (In vitro) and RPMI 1640 (In vitro) containing 10% FCS (Bio Whitaker, Verviers, Belgium). Human OVCAR-3 ovary adenocarcinoma cells (ATCC), hereafter called OVCAR, were cultured in RPMI 1640 (In vitro) containing 20% FCS. Human saphen vein endothelial cells were isolated from freshly excised vein as described elsewhere (28), and cultured in RPMI supplemented with 20% human serum and endothelial cell growth factor supplement. The BoHV-4 Vtest strain and a derived recombinant strain expressing EGFP called Vtest EGFP XhoI (23), were used throughout this study. The Vtest EGFP XhoI strain carries an enhanced green fluorescent protein (EGFP) expression cassette under the control of the human cytomegalovirus immediate-early promoter/enhancer inserted into a noncoding region of BoHV-4. BoHV-4 Vtest EGFP XhoI recombinant strain leads to EGFP expression in cells that are sensitive (supporting viral entry) and/or permissive (supporting viral replication) to BoHV-4 infection (23). Semipurified virus preparations prepared as described elsewhere (29) were used for cell inoculation.

Apoptosis assays. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) and Annexin V-FITC versus propidium iodide stainings were carried out with the In situ Cell Death Detection kit, TMR red (Roche, Mannheim, Germany), and the Annexin V-FLUSO Staining Kit (Roche), respectively. DNA laddering was done as described elsewhere (30).

Cell viability. Cell viability was assessed by Annexin V-FITC-propidium iodide staining as described elsewhere (29). The percentage of viable cells was defined as the percentage of cells excluding Annexin and propidium iodide staining as described elsewhere (23). Cell viability was assessed by Annexin V-FITC-propidium iodide staining as described elsewhere (29).

Viral photoinactivation by psoralen/UV treatment. Viral photoinactivation by psoralen/UV treatment. Semipurified virus preparations containing 5 × 106 viral plaque-forming units (pfu) were incubated in 1 mL of MEM containing 5 μg/mL psoralen (trioxysilen, 49-aminomethyl-HCl; Sigma, St Louis, MO) and incubated on ice in a 35 mm plastic Petri dish for various periods of time under UV light (302 nm, 0.12 A, UVM-57 lamp, Appleitek, Deinze, Belgium) at a distance of 5 cm. The efficiency of viral inactivation was monitored for each preparation by titration of residual infectivity on permissive Madin-Darby bovine kidney cells as described elsewhere (29).

Inhibition of viral gene expression. Subconfluent monolayers of A549 or OVCAR cells were infected with BoHV-4 Vtest strain at a multiplicity of infection (MOI) of 1 pfu per cell. From the time of infection up to the detection of apoptosis, cycloheximide (50 μg/mL) or phosphonoacetic acid (300 μg/mL) were added to the culture medium to inhibit de novo protein synthesis or DNA polymerase, respectively (32).

Inhibition of caspase activity. Caspase activity was inhibited using various caspase inhibitors (BioVision, Mountain View, CA): caspase-1 inhibitor, Z-YVAD-FMK; caspase-2 inhibitor, Z-LEHD-FMK; caspase-3 inhibitor, Z-DEVD-FMK; caspase-4 inhibitor, Z-LEVD-FMK; caspase-5 inhibitor, Z-WEHD-FMK; caspase-6 inhibitor, Z-VEID-FMK; caspase-8 inhibitor, Z-IETD-FMK; caspase-9 inhibitor, Z-LEHD-FMK; caspase-10 inhibitor, Z-AEVD-FMK; caspase-13 inhibitor, Z-LEED-FMK; and a pan-caspase inhibitor, Z-VAD-FMK. Z-Fa-FMK was used as a negative control. Cells were incubated at 37°C in the presence of these inhibitors from 2 hours before BoHV-4 infection up to the analysis of cell viability at 26 hours postinfection.

Cell membrane labeling. For long-term labeling, cellular membranes were loaded with the nontoxic lipophilic fluorescent marker PKH26 (Sigma) according to the instructions of the manufacturer.

Experimental animals. Female athymic nude (nu/nu) Naval Medical Research Institute mice at 6 weeks of age (JAVIER, Le Genest St Isle, France) were housed five per cage in polycarbonate filter–capped microisolation cages in temperature-controlled rooms maintained in a barrier facility on 12 hours light/dark cycles and provided with food and water ad libitum. Mice were ear-tagged so that data obtained from individual animals could be traced. The animal study done has been accredited by the local ethics committee.

Tumorigenicity experiments. A549 cells were infected with BoHV-4 Vtest strain at a MOI of 1. After an incubation of 2 hours, 106 infected tumor cells or mock-infected control cells were implanted s.c. into the right flank region of five nude mice. A549 xenograft treatment. A549 cells (107) resuspended in 100 μL PBS were implanted s.c. in the right flank region of nude mice. Tumor growth was measured every other day using a caliper. Tumor volume (V) was calculated using the equation V = (4/3)π(a/2)(b/2)2, where a is the largest diameter and b is the smallest diameter. When the average size of tumors reached 50 to 100 mm3, mice were randomly divided into two groups containing nine mice each. For the treated mice, BoHV-4 Vtest EGFP XhoI (107 pfu in 100 μL PBS) was injected into the tumors using a 27-gauge needle every other day over a period of 16 days. Control mice were injected with an equivalent volume of PBS. Differences in tumor volumes were tested in the form of a mixed model for repeated measurements with one main criterion, treatment (1 degree of freedom), nine replicates per treatment and 11 successive measurements per replicate. Correlation between successive measurements was modeled using a type 1 autoregressive structure (SAS, procedure MIXED).

Histochemical analysis. Tumor nodules were removed and weighed on day 21 postxenograft. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. Five-micrometer sections were stained with H&E, and either immunostained with monoclonal antibodies against Ki-67 (MIB-1 monoclonal antibody; Coulter Immunechn, Miami, FL) for detection of cell proliferation (33) or stained with TUNEL for detection of apoptosis (33). For quantification of both proliferation and apoptosis, the number of labeled nuclei per high power microscopic field was counted.

Flow cytometry. Flow cytometry analyses were done using a Recton Dickinson (Erembodegem, Belgium) fluorescent activated cell sorter (FacsAria) equipped with an argon ion laser (Innova Technology with 100 mW excitation line at 488 nm), as described elsewhere (34).

Confocal microscopy analysis. Confocal microscopy analyses were done with a TCS SP confocal microscope (Leica, Heerbrugg, Switzerland), as described previously (35).
Results

BoHV-4 induces apoptosis in A549 and OVCAR cell lines. During our recent investigation on the susceptibility of human cell lines to BoHV-4 infection (23), we observed that inoculation of BoHV-4 into A549 and OVCAR carcinoma cell lines resulted in a strong cytopathic effect suggestive of apoptotic cell death (data not shown). Here, to further investigate this observation, cytopathic BoHV-4–infected A549 and OVCAR cells were analyzed by three independent techniques for detection of apoptosis. First, early apoptotic stage was assessed 12 hours postinfection by detection of phosphatidylserine at the cell surface (Fig. 1A). For both A549 and OVCAR cultures, the percentages of apoptotic cells were significantly higher in BoHV-4–infected cells than in mock-infected cells. Second, internucleosomal DNA fragmentation associated with late apoptotic stages was analyzed 24 hours postinfection by TUNEL assay on cell monolayer (Fig. 1B); and third by electrophoretic analysis of cellular DNA (Fig. 1C). Both approaches confirmed that BoHV-4 induces apoptosis of OVCAR and A549 cells.

Apoptosis induced by BoHV-4 infection in OVCAR and A549 cells occurred in a time- and dose-dependent manner. To further characterize the induction of apoptosis by BoHV-4 in A549 and OVCAR cell lines, we first analyzed the kinetic of apoptosis appearance after BoHV-4 infection at a MOI of 1 pfu/cell (Fig. 2A). Apoptosis was monitored by Annexin V-FITC and propidium iodide labeling. Apoptosis was detected as early as 12 hours postinfection. Whereas at 12 hours postinfection most apoptotic cells were positive for Annexin V but negative for propidium iodide labeling, an increasing proportion of Annexin V–positive cells turned out to be also positive for propidium iodide labeling at later time points. The percentage of propidium iodide–positive Annexin V–negative cells was negligible throughout the experiment. Consequently, in this experiment and the following ones, rather than expressing the percentage of apoptotic cells (cells positive for Annexin V but negative for propidium iodide labeling), the data will be expressed as the percentage of viable cells (cells negative for Annexin V and propidium iodide labeling). The percentage of viable cells reduced gradually over time up to the end of the experiment to reach 13% and 22% in OVCAR- and A549-infected cultures, respectively. The detection of apoptotic cells as early as 12 hours postinfection suggested that an early event of the viral infection, such as the binding of the virion on the cell surface, acts as a proapoptotic signal. In herpesvirus preparations, ~99% of physical particles are not infectious (36). To determine whether the proapoptotic signal was delivered by all physical particles or only by infectious particles, we next investigated the effect of the MOI used for the inoculation on the rate of viable cells in the culture (Fig. 2B). If all physical particles are able to deliver the proapoptotic signal, the induction of apoptosis should be observed with MOI lower than 1. For both cell types, a clear correlation was observed between the percentage of viable cells measured 36 hours postinfection and the reduction of the MOI used for the inoculation. Infection done at a MOI of 0.01 pfu/cell led to a proportion of viable cells comparable with mock-infected cells. These data suggested that induction of apoptosis by BoHV-4 in A549 and OVCAR cells relies on an early event of the infection mediated only by infectious particles.

Induction of apoptosis by BoHV-4 in A549 and OVCAR cells requires viral gene(s) expression. The results presented above suggest that viral gene(s) expression or even viral replication could be required for induction of apoptosis in A549 and OVCAR cells. To investigate this hypothesis, BoHV-4 Vtest or Vtest EGFP Xhol

Figure 1. BoHV-4 infection induces apoptosis of OVCAR and A549 cells. A, phosphatidylserine translocation associated with early stages of apoptosis was detected by Annexin V-FITC and propidium iodide labeling. OVCAR and A549 cells were mock infected or infected with the BoHV-4 Vtest strain at a MOI of 1 pfu/cell. After an incubation period of 12 hours, the cells were harvested, stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. Cellular DNA fragmentation associated with late stages of apoptosis was detected by two independent techniques 24 hours after infection. B, TUNEL reaction. Mock-infected and infected cells grown on glass coverslips were submitted to TUNEL staining and were analyzed by confocal microscopy. The longer side of each panel corresponds to 500 μm of the specimen. C, DNA laddering. Cellular DNA extracted from mock-infected and infected cultures was analyzed by electrophoresis to detect DNA laddering. Marker sizes (MS) in kbp are indicated on the left.
Virions were submitted to a treatment associating incubation with a psoralen compound and exposure to UV light (Fig. 3A and B). This treatment provokes DNA cross-linking in the viral genome proportionally to time of UV exposure without affecting viral structural proteins (31). Whereas short UV exposure responsible for small amounts of DNA cross-linking anywhere in the viral genome is sufficient to prevent viral DNA replication, and consequently production of progeny virions, total inhibition of viral gene expression requires longer UV exposure inducing cross-linking throughout the genome. In theory, the probability of inhibiting the expression of a gene is related to its size, longer genes being inactivated before shorter genes. The effect of psoralen UV inactivation on BoHV-4 was monitored by plaque assays. UV exposure as short as 2 minutes in the presence of psoralen reduced BoHV-4 infectivity by 5 log, whereas exposure of 4 minutes or longer led to complete inactivation of infectivity (starting with 5 × 10^7 pfu). Interestingly, incubation of the virus with psoralen for 8 minutes in the absence of UV or exposure of the virus to UV for 8 minutes in the absence of psoralen did not affect significantly the titer of the viral preparation. The results presented in Fig. 3A reveal that BoHV-4 treated with psoralen/UV for 2 minutes retains its ability to induce apoptosis despite the drastic reduction of the viral titer. This observation shows that BoHV-4 induction of apoptosis in OVCAR and A549 cells does not require viral replication. The viability of cell cultures infected with BoHV-4 treated with psoralen/UV for 8 minutes was similar to mock-infected cultures (Fig. 3A). Interestingly, examination by confocal microscopy of cells infected with the BoHV-4 Vtest EGFP XhoI strain treated similarly revealed the expression of EGFP by a large proportion of infected cells (Fig. 3B). This observation shows that psoralen/UV-inactivated virions still retain their property to enter cells, and consequently that the apoptosis induced by BoHV-4 does not rely upon the delivery of structural proteins into the cell, but rather upon the expression of some viral gene(s). Taken together, the data presented above suggest that the observed induction of apoptosis by BoHV-4 requires the expression of some viral gene(s) that are longer or more accessible to psoralen/UV inactivation than the EGFP reporter gene.

Next, to determine the kinetic class of the BoHV-4 gene(s) inducing apoptosis in A549 and OVCAR cells, infected cells were treated with cycloheximide or phosphonoacetic acid to inhibit de novo protein synthesis or viral DNA polymerase, respectively (Fig. 3C). These effects were controlled on infected Madin-Darby bovine kidney cells by indirect immunofluorescent staining with antibodies raised against early and late proteins, as described previously (ref. 23; data not shown). The results presented in Fig. 3C reveal that, in contrast to phosphonoacetic acid, cycloheximide was able to suppress cell death induced by BoHV-4 in A549 and OVCAR.

Figure 2. Apoptosis induced by BoHV-4 infection in OVCAR and A549 cells occurred in a time-dependent (A) and dose-dependent (B) manner. A, OVCAR and A549 cells were mock infected or infected with the BoHV-4 Vtest strain at a MOI of 1 pfu/cell. At the indicated time after infection, cell viability was assessed by Annexin V-FITC and propidium iodide labeling and flow cytometry analysis. B, OVCAR and A549 cells were mock infected or infected with the BoHV-4 Vtest strain at the indicated MOI. After an incubation period of 36 hours, cell viability was assessed as described above. Columns, mean percentages of viable cells from triplicate experiments; bars, 2 SD.
cell cultures. This observation suggests that the BoHV-4 gene(s) inducing the observed apoptosis belong(s) to the immediate-early or early kinetic class of expression.

Apoptosis induced by BoHV-4 in A549 and OVCAR cells is dependent on caspase-10 activity. To investigate the implication of caspases in the apoptosis induced by BoHV-4 in A549 and OVCAR cells, experiments were done with various inhibitors of these proteases (Fig. 4). First, the property of the pan-caspase inhibitor Z-VAD-FMK to block BoHV-4–induced apoptosis was tested (Fig. 4A). In both cell types, the pan-caspase inhibitor Z-VAD-FMK inhibited cell death induced by BoHV-4 in a dose-dependent manner; however, the inhibition was more efficient in OVCAR cells. These results show that the observed apoptosis induced by BoHV-4 requires the activation of caspases. Second, with the goal of identifying specific caspases involved in this process, we investigated the effect of 10 specific caspase inhibitors. The results of these experiments are presented in Fig. 4B and are expressed as the percentage of inhibition of apoptosis observed with the pan-caspase inhibitor Z-VAD-FMK. Among the inhibitors tested, Z-AEVD-FMK, inhibiting specifically caspase-10, reduced BoHV-4–induced apoptosis in a similar way to the pan-caspase inhibitor Z-VAD-FMK (Fig. 4B). Taken together, the results presented above suggest that induction of apoptosis by BoHV-4 in A549 and OVCAR cells is a caspase-dependent process and that the most apical caspase activated during this process is caspase-10.

BoHV-4 selectively induces apoptosis of A549 and OVCAR cells in cocultures. The results presented above suggest that BoHV-4 could be a candidate for viro-oncoapoptotic treatment of some human carcinomas. To test this possibility, we investigated the property of BoHV-4 to induce selectively the death of A549 and OVCAR cells cocultivated with human 293T cells, in which BoHV-4 does not induce apoptosis (Fig. 5). The results presented in Fig. 5 show that infection of the cocultures led to induction of apoptosis in OVCAR and A549 cells but not in 293T cells of the same
The percentage of 293T cells in the cocultures was investigated over a period of 72 hours after infection. The results obtained show that BoHV-4 infection drastically increased the percentage of 293T cells in the cocultures. To further investigate the specificity of BoHV-4 proapoptotic signal for OVCAR and A549 cells, the impact of BoHV-4 infection was investigated on non-transformed human saphen vein endothelial cells. Forty-eight hours postinfection at a MOI of 1, the percentages of apoptotic cells were similar in BoHV-4–infected cells compared with mock-infected cells (data not shown). Taken together, these results support the potential of BoHV-4 as a candidate for viro-oncoapoptotic treatment of some cancers.

BoHV-4 abolishes the tumorigenicity of A549 cells. To further investigate the potential of BoHV-4 as a candidate for viro-oncoapoptotic treatment of human carcinomas, in vitro experiments were done following classic approaches described elsewhere (37). All the in vitro experiments described above were done in parallel with OVCAR and A549 cells. As both cell lines led to identical conclusions, it can be assumed that the two cell lines are reflecting the same phenomenon. Consequently, the in vivo experiments described below were pursued for economical and bioethical reasons with only one of the two cell lines. The A549 cell line was selected arbitrarily.

Figure 4. Apoptosis induced by BoHV-4 in A549 and OVCAR cells is dependent on caspase-10 activity. A, apoptosis induced by BoHV-4 is a caspase-dependent process. OVCAR and A549 cells were mock infected or infected with the BoHV-4 Vtest strain (MOI of 1 pfu/cell) and cultured for 24 hours in the presence of growing doses of the pan-caspase inhibitor Z-VAD-FMK. Cell viability was assessed by Annexin V-FITC and propidium iodide labeling and flow cytometry analysis. Columns, mean percentages of viable cells from triplicate experiments; bars, 2 SD. B, effect of caspase-specific inhibitors on BoHV-4–induced apoptosis. A549 and OVCAR cells were infected at a MOI of 1 pfu/cell with the BoHV-4 Vtest strain and further cultured for 24 hours in the presence of 4 μmol/L (OVCAR) or 20 μmol/L (A549) of various caspase-specific inhibitors. The properties of each inhibitor to inhibit totally (+++) or partially (+) specific caspases are indicated. Z-VAD-FMK and Z-FA-FMK were used as positive and negative control, respectively. Apoptosis was assessed by Annexin V-FITC and propidium iodide labeling and flow cytometry analysis. Inhibition of apoptosis by each specific inhibitor is expressed as a percentage of the inhibition induced by the pan-caspase inhibitor Z-VAD-FMK. Columns, mean of triplicate experiments; bars, 2 SD.
developed macroscopic tumors within 2 weeks after injection. They were euthanized for bioethical reasons ~1 month after injection when the mean volume of the tumors reached 1,000 mm³. At the opposite, none of the five mice injected with BoHV-4–infected cells developed tumors over a period of 3 months after implantation. This result suggests that, among the A549 cell population, no cells are resistant to the apoptosis induced by BoHV-4.

**Intratumoral BoHV-4 injections cause regression of established A549 xenografts.** Next, we tested the potential of intratumoral BoHV-4 injection as a treatment of tumors preestablished in nude mice (Fig. 6). Ten million A549 cells were injected s.c. in the right flank of nude mice. After 5 days, cell proliferation led to the formation of a macroscopic tumor with a diameter of ~5 mm. Starting at day 5, tumors were injected with 100 μL PBS or with 100 μL PBS containing 10⁸ pfu of semipurified BoHV-4 Vtest EGFP XhoI strain. Injections were done every other day over a period of 16 days. At day 21, all mice were euthanized and the tumors were harvested for further analysis. As shown in Fig. 6A, from day 15, there was a significant difference in the rate of progression of BoHV-4 injected tumors compared with controls. This effect was objectified by the dissection of the tumors at the end of the experiments and by weighing them (Fig. 6B). Tumors injected with BoHV-4 or control PBS exhibited a mean weight of 86 and 264 mg, respectively. Histopathologic analysis of the

![Figure 5. Induction of apoptosis by BoHV-4 in cocultures of OVCAR or A549 cells mixed with 293T cells. OVCAR or A549 cells were mixed with PKH26-labeled 293T cells at the ratio of 70:30. The cocultures were mock infected or infected with the BoHV-4 Vtest strain at a MOI of 1 pfu/cell. At the indicated time after infection, the cells were washed or harvested, stained with Annexin V-FITC, and finally analyzed by flow cytometry for FITC and PKH26 fluorescence. In each dot plot, the percentage of apoptotic cells were estimated for each cell types (e.g., the percentage of A549 or OVCAR apoptotic cells was calculated as the ratio of the number of cells in upper left quadrant divided by the total number of cells in left quadrants). In each dot plot, the percentages of apoptotic cells are presented in italic for each cell line. The percentages of 293T cells in the cocultures were estimated by flow cytometry based on PKH26 fluorescence; they are indicated on the top of each dot plot.](Image)
sensitivity of cells deriving from BoHV-4–or PBS-treated tumors to BoHV-4–induced apoptosis and that the complete eradication of tumor cells requires injection of a higher titer of the virus and/or more frequent injections.

**Discussion**

The extreme diversity of cancer cells requires the development of complementary therapeutic approaches acting as independent pressures of selection on malignant cells. Recently, a new approach consisting of inducing selective apoptosis in cancer cells through viral infection has been proposed; this approach has been called viro-oncoapoptosis (4). The beneficial effect of viro-oncoapoptosis relies on the induction of cancer cell death (like classic viro-oncolysis) but also on its potential to induce a tumor specific CD8 T-cell response. Indeed, recent studies have shown that tumor cell death by apoptosis may lead to MHC-I tumor antigen cross-presentation by antigen presenting cells that have internalized apoptotic bodies. This cross-presentation has been shown to induce cross-priming rather than cross-tolerizing host tumor–specific CD8 T cells (38). In the present study, we report the property of BoHV-4 to induce apoptosis in two human carcinoma cell lines. This phenomenon relied on the expression of immediate-early and/or early BoHV-4 gene(s) and involved caspase-10 activation. *In vitro* and *in vivo* experiments revealed the potential of BoHV-4 as a candidate for viro-oncoapoptotic treatment of some human cancers.

BoHV-4 induces apoptosis of human carcinoma OVCAR and A549 cells. In our recent study on the susceptibility of human cells to BoHV-4 infection, 21 human cell lines were tested for their sensitivity and their permissiveness (23). These experiments revealed that human cell lines from lymphoid and myeloid origins were resistant to infection, whereas epithelial cells, carcinoma, or adenocarcinoma cells isolated from various organs were sensitive but poorly permissive to BoHV-4 infection. Despite their sensitivity to the infection, BoHV-4 did not induce apoptosis in those cell lines. To date, none of our data can explain or suggest why OVCAR and A549 are sensitive to BoHV-4–induced apoptosis, whereas the other cell lines tested are not. This restriction could be the consequence of the expression of a particular viral and/or cellular proteome. Microarray experiments to address this hypothesis are in progress. These experiments could also explain why BoHV-4 induces apoptosis in OVCAR and A549 cells, whereas persistent nonpermissive infection of HeLa cells protects them from TNF-α–induced apoptosis. Our recent unpublished data showed that the latter observed effect was due to BoHV-4 encoded v-FLIP.

Induction of apoptosis by BoHV-4 in OVCAR and A549 cell lines relies on the expression of immediate-early and/or early BoHV-4 genes (Fig. 3). Interestingly, an immediate-early or early gene product of the relatively close γ-herpesvirus, human herpesvirus 4, has also been shown to induce apoptosis in some cell lines (39). Further studies are required to identify the proapoptotic genes of these γ-herpesviruses and to determine if encoding proapoptotic proteins is a general property of this subfamily. At first sight, it is surprising to observe that BoHV-4 gene expression is able to induce apoptosis in some cell types, whereas BoHV-4 is principally known to encode antiapoptotic genes (24, 25). Such paradoxes have been
shown for other herpesviruses (40, 41). Recently, Hood et al. (41) provided evidence for a cell type–specific apoptotic response to VZV infection. They postulated that viral interference with apoptotic pathways is different at the different stages of the infection. From a viral point of view, inhibition of apoptosis could maximize the production of virus progeny during lytic infection or could facilitate a persistent infection. Alternatively, apoptosis could also be actively induced a latter point of the replication cycle by some viruses as a mechanism for efficient dissemination of progeny virus. Until now, studies on viral interference with apoptosis have been mainly focused on apoptosis inhibition, perhaps owing to the implication of these phenomena in cancer development. In the future, studies on how viruses trigger apoptosis and identification of the viral gene products involved in these phenomena should lead to the discovery of new cellular apoptotic pathways in the same way that the study of apoptosis inhibition by viruses led to our understanding of how cells negatively regulate apoptosis. Indeed, it was the discovery of herpesvirus v-FLIP (42), for example, that led to the characterization of cellular c-FLIP (43).

From the cellular point of view, this study shows that BoHV-4 induces apoptosis by a pathway involving caspase activation. More precisely, specific caspase inhibitors pointed to caspase-10 as the most apical caspase activated in A549 and OVCAR cells following BoHV-4 infection. Caspase-10 has been proposed mainly as an initiator caspase thought to be an analogue of caspase-8, based on sequence homology and on the presence of death effector domains (44). However, little is known of its activation stimulus or cellular substrates. As for caspase-8, caspase-10 activation is likely to occur at the death receptor level through interaction with the death effector domains. It is usually thought that the binding of death receptors to their ligands results in recruitment and activation of caspase-8 and caspase-10, formation of the death-inducing signaling complex, and subsequent initiation of the apoptotic cascade. In accordance with this model, several viral infections have been shown to activate caspase-8 and/or caspase-10 by strongly up-regulating the expression of death ligands, such as FAS or TNF-related apoptosis-inducing ligand, or even by simultaneously up-regulating the expression of their specific death receptors (45). Independently, it has been shown that formation of reactive oxygen species can promote the clustering of death receptor FAS and, thus, death-inducing signaling complex formation (46). Interestingly, we recently observed reactive oxygen species formation in A549 cells. Preliminary results suggest that, in A549, reactive oxygen species formation induced by BoHV-4 infection precedes caspase activation. Further experiments will be needed to investigate the possible involvement of reactive oxygen species formation in caspase activation after BoHV-4 infection of A549 or OVCAR cells, and to understand the cellular pathways exploited by the virus.

Future studies on the identification of the proapoptotic BoHV-4 gene product(s) involved in this phenomenon, or eventually the development of BoHV-4 as a viro-oncoapoptotic vector, will be greatly facilitated by the recent bacterial artificial chromosome cloning of BoHV-4 (47). By using random transposon mutagenesis, a library of mutant viruses could be generated with minimal effort. This approach may result in the identification of the proapoptotic BoHV-4 gene(s). In addition, BoHV-4 bacterial artificial chromosome cloning will facilitate a possible development of BoHV-4 as a new viro-oncoapoptotic vector. The safety of BoHV-4 could be improved by the deletion of genes responsible for deleterious effects, such as ORF71 encoding BoHV-4 v-FLIP, conferring protection to TNF-α induced apoptosis. Deletion of those genes, among others, could improve the safety and the cloning capacity of this vector. We recently showed that BoHV-4 allows the stable insertion of additional genetic material in its genome of up to at least 10.5 kb. This relatively large cloning capacity, combined with the simplicity of recombination in bacteria, offers the possibility of generating a panel of viruses able to deliver therapeutic factors (armed therapeutic viruses) to attack more effectively the complexity of tumors. Indeed, although this study shows that intratumoral BoHV-4 injections are able to reduce the growth of established A549 tumor xenografts, it also shows that complete regression was not reached, probably owing to a lack of virus dissemination into the tumor and/or to the administration of insufficient virus doses. The insertion of additional therapeutic genes into the BoHV-4 genome could solve this problem by developing a bystander effect, as shown in other viruses (48).

In conclusion, this study shows that the expression of some immediate-early or early BoHV-4 genes induces apoptosis of some human carcinoma cell lines through a pathway involving caspase-10 activity. In vitro and in vivo experiments showed the potential of BoHV-4 as a new candidate for the development of a viro-oncoapoptotic vector.

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